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Altered Expression of Proliferation and Differentiation Markers in Human Papillomavirus 16 and 18 Immortalized Epithelial Cells Grown in Organotypic Culture

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The patterns of expression of keratins K1 and K8, filaggrin, and the proliferation-associated protein, proliferating cell nuclear antigen (PCNA), were studied in normal and human papillomavirus (HPV) 16 or 18 immortalized keratinocyte cell lines grown on organotypic raft cultures. Normal keratinocytes produced an epithelial sheet that closely resembled epidermis in vivo, ***characterized by lack of K8 expression, PCNA expression restricted to the basal layer, and K1 and filaggrin expression in the suprabasal layers. Although morphologically abnormal in many respects, some HPV-immortalized cell lines produced cornified epithelial layers and approximated the normals in their patterns of expression of keratins and filaggrin. Other HPV-immortalized cell lines produced poorly differentiated epithelial layers that were characterized by loss of filaggrin expression, and the single tumorigenic cell line, 18-11, was distinguished by abundant K8 expression. All of the HPV-immortalized cell lines were distinguished from normal keratinocytes by a common pattern of full-thickness PCNA expression in the epithelial layers they produced, suggesting that maintenance of the proliferative state may be an important contribution made by HPV 16 or 18 sequences toward the production of a malignant phenotype. (Am J Pathol 1992, 140:167–177)***

Human papillomaviruses (HPV) are associated with lesions of the epithelium, and several HPV types have been implicated as causative agents in cervical and other epithelial malignancies. Nearly all cervical cancers contain HPV sequences, mostly from types 16 or 18.[1,2] Cells derived from these malignancies contain transcriptionally active HPV E6 and E7 open reading frames (ORFs), whereas the rest of the viral genome may be either absent or inactive.[3–5] Additionally the development of certain other malignancies, such as the epidermal squamous cell carcinomas that develop in patients with epidermodysplasia verruciformis, have been linked to infection with other specific HPV types.[6] Products from the E6 and E7 ORFs of HPV types 16 and 18 have been shown to form complexes with the normal cellular proteins p53 and the retinoblastoma gene product Rb.[7,8] It has been suggested that the effects these complexes have on cell cycle control could contribute to malignant progression in cells harboring HPV sequences.[7–10] Many current studies are aimed at further defining the characteristics of cells expressing HPV genes that progress to the malignant state.

Epithelial cells can be immortalized *in vitro* by introducing stably expressed E6 and E7 ORFs from HPV 16 or 18.[11–15] Human papillomavirus–immortalized cells infrequently will become tumorigenic after passage in tissue culture,[16,17] but generally are not malignant unless additional steps are taken, such as introduction of an activated *ras* oncogene.[18] Several *in vivo* studies of cervical lesions suggest that dysplasia can progress to malignancy,[19,20] and therefore often represent premalignant lesions. Previously reported isolation of immortalized HPV-16–containing keratinocytes from a premalignant cervical lesion[21] support the idea that HPV-immortalized keratinocytes provide an *in vitro* model of premalignant cells. Also growth of HPV-immortalized cell lines on organotypic cultures produces epithelial layers that closely resemble these premalignant lesions.[22–24] Some phenotypic changes that might differentiate cervical cancers and their premalignant lesions from other benign lesions and normal epithelium are beginning to be identi-

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fied,[25–29] and organotypic cultures of HPV-immortalized cell lines can provide *in vitro* verification of these phenotypic alterations. The role HPV plays in the development of premalignant lesions also can be addressed in such studies.

We have looked at the expression of a number of epithelial markers in organotypic cultures of several cell lines that were previously immortalized by transfection of HPV 16 and 18 DNA sequences.[12–14] The organotypic cultures, in which epithelial cells are seeded on a dermal equivalent and grown at the air–liquid interface, allow stratification and epithelial differentiation that closely resembles normal epithelia.[22,24,30,31] These cell lines produce a variety of morphologic changes when grown in organotypic cultures, as previously characterized.[22] Some of the cell lines have retained the ability to cornify and produce epithelial sheets that are more differentiated, whereas others give highly disorganized epithelial layers that resemble carcinoma *in situ*.[22] In addition, the tumorigenic potential of these cell lines has been defined, and one cell line, 18-11, has been shown to be tumorigenic in nude mice.[16] This system allows comparison of the HPV-immortalized cell lines with normal epithelial cells grown in organotypic culture, and allows us to characterize the cell line's terminal differentiation capabilities and other phenotypic abnormalities.

The degree of differentiation of each organotypic culture was assessed by studying the levels of expression of filaggrin and keratins K1 and K8. The expression of the S-phase–associated protein proliferating cell nuclear antigen (PCNA) was used to study the proliferative state of each cell line. Our results show that specific abnormalities in PCNA expression are correlated with the immortalized phenotype, and that different patterns of keratin and filaggrin expression can be correlated with the morphologic characteristics and tumorigenic potential of each cell line.

Materials and Methods

Culture of Foreskin Keratinocytes and Fibroblasts

Normal human foreskin keratinocyte and fibroblast monolayer cultures were established and maintained as previously described.[22] Briefly, foreskins were incubated overnight at 4°C with 25 mg/ml dispase (Boehringer-Mannheim, Indianapolis, IN) prepared in phosphate-buffered saline (PBS). The epidermis was removed with forceps, minced, trypsinized, washed in PBS, pelleted at 1000*g* for 5 minutes, and cultured in monolayer in keratinocyte-SF medium (Gibco, Grand Island, NY). The dermis was minced, digested with 25 mg/ml collagenase (Worthington, Freehold, NJ) at 37°C for 30 to 60 minutes, pelleted at 1000*g* for 5 minutes, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.4 μg/ml hydrocortisone. Primary epidermal and fibroblast cultures were trypsinized and split at a ratio of 1:3 at confluence.

Immortalized Foreskin Keratinocyte Cell Lines

The HPV-immortalized cell lines used in this study were originally isolated in our laboratory after transfection of HPV 16 or 18 DNA sequences into normal primary human foreskin epithelial cultures.[12–14] These cells lines include the HPV-16–containing FEPEIL 8 and FEPEIL 13 cell lines, and the HPV-18–containing 18-5, FEA, and 18-11 cell lines. The presence and physical state of the HPV sequences in these cell lines has been confirmed and characterized in earlier publications,[12–14,32] and their growth and differentiation properties in organotypic cultures has been described previously.[22] These cell lines were grown and maintained in keratinocyte—SFM® (Gibco), which was changed every 2 days, and they were split at a ratio of 1:3 at confluency. The passage levels of the cells used in organotypic cultures ranged from passage 60 to 100.

Organotypic Raft Cultures

Organotypic cultures were generated as previously described.[22] Briefly, dermal equivalents were constructed with bovine dermal collagen (Collaborative Research, Bedford, MA), a reconstitution buffer (consisting of 2.2% $NaHCO_3$, 0.05 N NaOH, and 200 mmol/l [millimolar] HEPES), and fibroblasts suspended in FBS. The dermal equivalents were allowed to solidify at 37°C for 2 to 4 hours, and then 5×10^5 cells of the various normal and immortalized keratinocytes were suspended in 2 ml organotypic growth medium and seeded on top of the gels. Organotypic growth medium consists of DMEM plus 10% delipidized FBS supplemented with hydrocortisone, tri-iodothyronine, epidermal growth factor, cholera toxin, transferrin, and insulin as described by Wu et al.[33] The FBS was delipidized with a silica-based reagent (reagent and protocol courtesy of International Enzymes Inc., Fallbrook, CA). The reagent was mixed with FBS at room temperature for 30 to 45 minutes, and the lipid fraction was removed by pelleting at 15,000*g* for 30 minutes. The supernatant was added to DMEM and the solution was filtered. Retinoic acid (Sigma, St. Louis, MO) then was added back to a final concentration of 10^{-8} mol/l (molar). Rafts were cultured while submerged 4 to 5 days, and

then raised onto stainless steel grids and allowed to grow at the air–liquid interface for 12 to 14 days. Medium was changed every 1 to 2 days. Cultures then were fixed in 100% methanol and embedded in paraffin.

Immunohistochemistry

Five-micron sections of the raft specimens were deparaffinized in xylene and rehydrated in graded alcohol incubations. Serial sections were incubated with the panel of monoclonal antibodies as described in Table 1. Localization of the antibodies was by a modification of the avidin biotin (ABC) immunoperoxidase method as previously described, in which nickel chloride is added to the chromogen 3,3′-diaminobenzidine to yield a black reaction product,[34] or, in the case of the anti-PCNA antibody, a modified streptavidin biotin immunoperoxidase technique, as previously described.[35]

Results

Morphology of Normal and Immortalized Foreskin Keratinocytes on Organotypic Cultures

Previous studies from our laboratory have demonstrated that HPV-immortalized cell lines can display a wide variety of morphologies on organotypic cultures.[26] Cell lines that represent the entire spectrum of differentiation potentials were selected for this study, and representative organotypic cultures of these selected cell lines are shown in Figure 1. Normal human foreskin epithelial cells (HFEs) grown on organotypic culture produce an epithelial sheet that closely resembles normal epidermis *in vivo*[22,24,30,31] with well-defined basal (B), spinous (S), granular (G), and cornified (C) layers (Figure 1). Human papilloma virus–immortalized FEPEIL 13 and 18-5 cell lines form cornified layers on organotypic cultures (Figure 1) and are more like HFEs than the other HPV-immortalized cell lines. The morphology of the FEPEIL 13 and 18-5 organotypic cultures is abnormal in a number of ways, however. The basal layer is expanded and the number of keratohyalin granules are reduced compared with the HFEs. Also the cornified layers formed by the FEPEIL 13 and 18-5 cell lines show a significant amount of parakeratosis. In contrast, organotypic cultures of the HPV-immortalized FEPEIL 8, FEA, and 18-11 cell lines show a different morphology characterized by a total lack of differentiation (Figure 1). None of the distinct layers of normal epidermis are distinguishable, and no cornification occurs. The epithelial sheet is disorganized and is composed of small dysplastic cells that display a high nuclear-to-cytoplasmic ratio. These cell lines produce epithelial layers that are undifferentiated, whereas the organotypic cultures of the FEPEIL 13 and 18-5 cell lines are more differentiated, producing a morphology between that of HFEs and the FEPEIL 8, FEA, and 18-11 cell lines. It is also important to note that the 18-11 cell line is the only cell line in this study that forms tumors in nude mice.[16]

Keratin 1 Expression

Organotypic cultures of these cell lines were sectioned and stained for the terminal squamous keratin K1, and results of these studies are shown in Figure 2. K1 expression in normal epidermis begins in the spinous layer and increases throughout the upper layers, where it is ultimately incorporated in the filaments that make up the majority of the cell in the cornified layer.[33,36–39] K1 expression in the organotypic culture of HFEs shown in Figure 2 closely resembles that seen previously in organotypic cultures and *in vivo*.[30,31,40] Strong K1 expression also can be seen in the organotypic cultures of FEPEIL 13 and 18-5 (Figure 2), although the thickness of the layer of unstained cells in the lower portion of the epithelial layer is larger than that seen with the HFEs. The FEA and FEPEIL 8 cell lines also show some reactivity with the anti-K1 antibody (Figure 2), but expression is not as marked as in the normal or cornifying FEPEIL 13 and 18-5 cell lines. The pattern of staining in these cell lines is also highly abnormal, with positive and negative cells intermixed throughout the upper epithelial layers. The 18-11 cell line

Table 1. *Antibodies Used in Immunocytochemical Analyses*

Antibody designation	Specificity	Source/Reference	Working dilution
35βH11	CK8	This laboratory (62)*	1:1000‡
34βB4	CK1/10	This laboratory (62)†	1:500‡
AKH1	Filaggrin	This laboratory (43)	1:500‡
19A2	PCNA	Coulter	1:8000‖

* Available from Dako Corporation, Carpinteria, CA.
† Available from Enzo Biochem, Inc., New York, NY.
‡ ABC method.
‖ Streptavidin-biotin method.

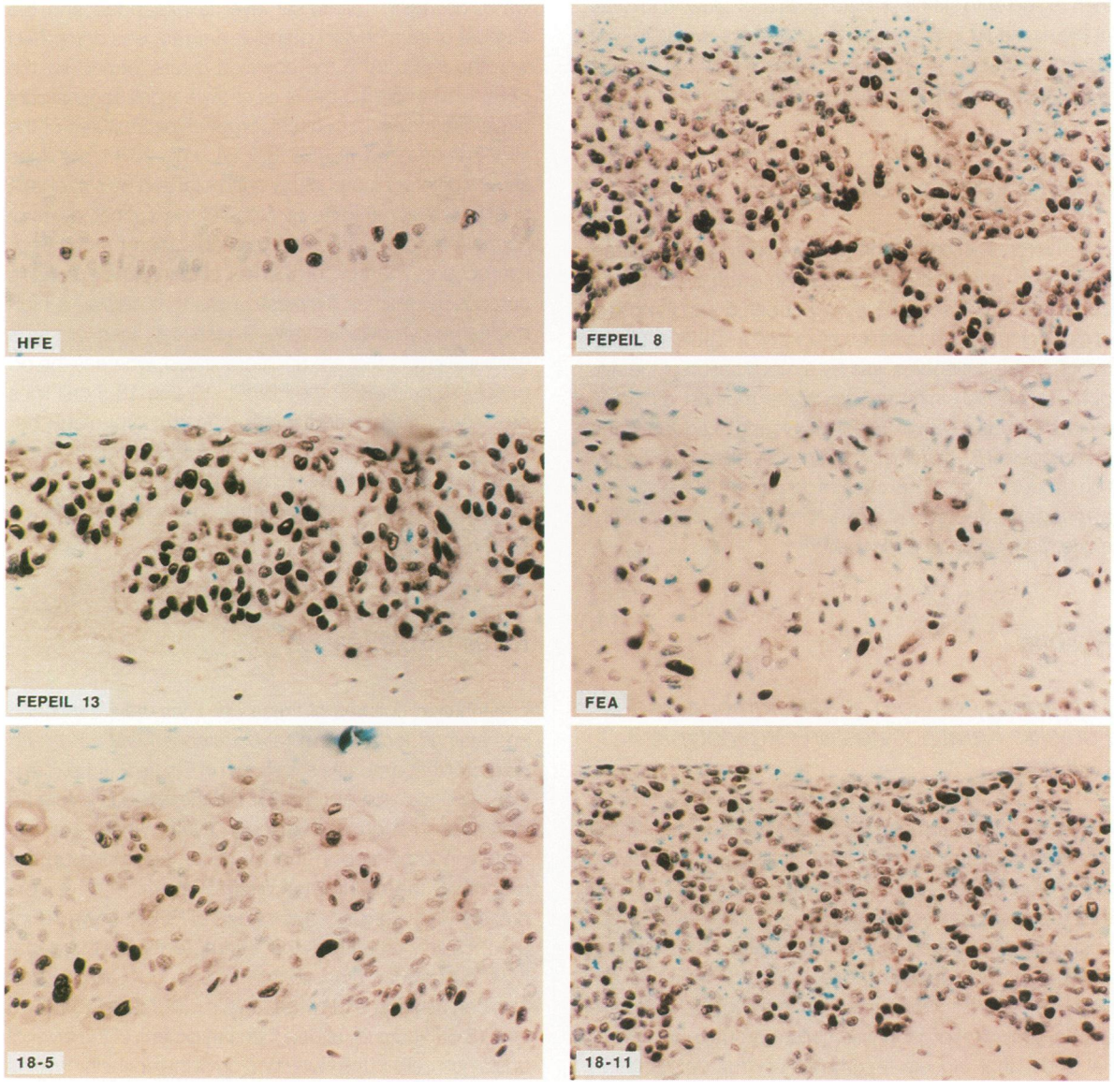

Figure 1. *Cross section of methanol-fixed, paraffin-embedded organotypic cultures of normal human foreskin epidermal cells (HFE) and HPV immortalized keratinocyte cells stained with H&E. In HFE panel: B = basal layer; S = spinous layer; G = granular layer; C = cornified layer, original magnification, ×200.*

grown on organotypic culture appears to completely lack K1 expression (Figure 2). Therefore it appears that decreased expression or lack of expression of keratin K1 is seen in all of the cell lines that lack terminal differentation in organotypic culture.

Keratin 8 Expression

Keratin 8 is a keratin that normally is expressed only in simple epithelia.[33,37] In normal epidermis[33,37] and in HFEs grown on organotypic culture, shown in Figure 3, there is no expression of keratin K8. The keratinizing cell lines FEPEIL 13 and 18-5 do not express K8 in organotypic culture either (Figure 3). The FEPEIL 8 cell line shows occasional weak positively stained cells, and the FEA cell line shows a more diffuse weakly positive staining pattern with the K8-specific antibody (Figure 3). The 18-11 cell line, however, expresses high levels of K8 (Figure 3) with a strongly positive and diffuse staining pattern involving the entire thickness of the epithelial layer of the organotypic culture. In summary, K8 expression occurs at least to a minimal degree in all of the cell lines that produce undifferentiated epithelial layers in organotypic culture, but is strongly expressed only in the tumorigenic 18-11 cell line.

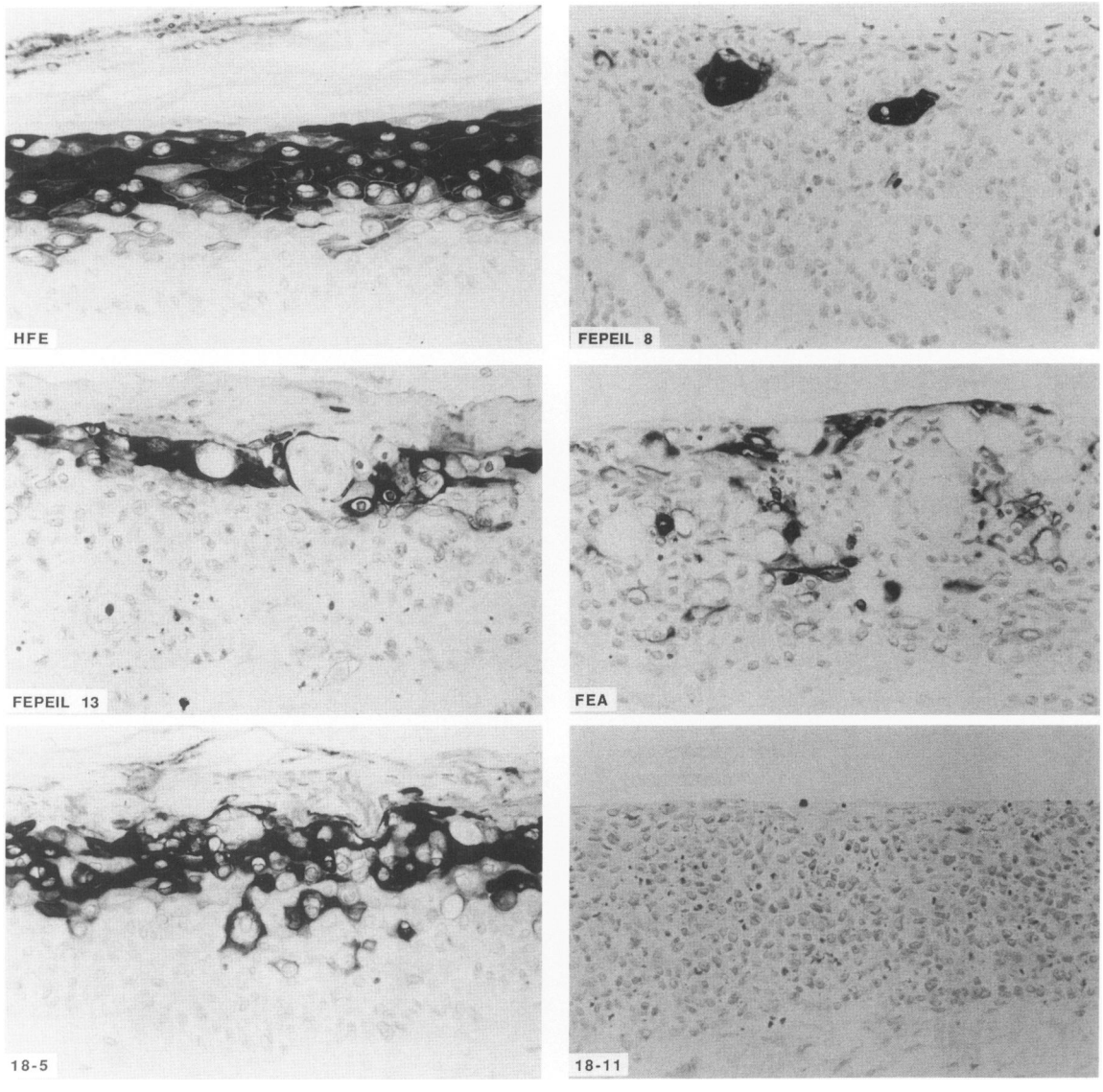

Figure 2. *Cross section of methanol-fixed, paraffin-embedded organotypic cultures of HFE and HPV immortalized keratinocytes. Immunohistochemistry of cytokeratin K1 expression with methyl green counterstain, original magnification, ×200.*

Filaggrin Expression

Filaggrin is normally expressed in the upper layers of squamous epithelia,[41–43] and it is thought to be necessary in the epidermis for the cross-linking of keratins that occurs in cellular cornification.[42,44,45] In organotypic cultures of normal HFEs, filaggrin expression closely resembles that seen *in vivo* (Figure 4).[42] Although the expression of filaggrin in the organotypic cultures of FEPEIL 13 and 18-5 is not quite as strong as that seen in the HFEs, it has the same general distribution as seen in the normals (Figure 4). In the nonkeratinizing FEPEIL 8, FEA, and 18-11 cell lines, there is no discernible filaggrin expression (Figure 4). Thus loss of filaggrin expression occurs specifically in the cell lines that do not terminally differentiate on organotypic cultures.

PCNA Expression

Proliferation and PCNA expression is normally confined to the basal layer of epithelia[46] (A. Gown unpublished results). PCNA is an auxiliary protein of DNA polymerase delta, and its expression is associated with the S phase of the cell cycle.[35,47,48] Proliferating cell nuclear antigen expression in organotypic cultures of HFEs is confined to the basal layer (Figure 5). In organotypic cultures of all of the HPV-immortalized cell lines, PCNA expression is present throughout the entire epithelial layer (Figure 5). There are some slight variations in intensity of staining between the cell lines, but the overall pattern of expression is quite similar in all of them. There is no notable difference in PCNA expression between the cornifying and noncornifying cell lines or between the tumorigenic

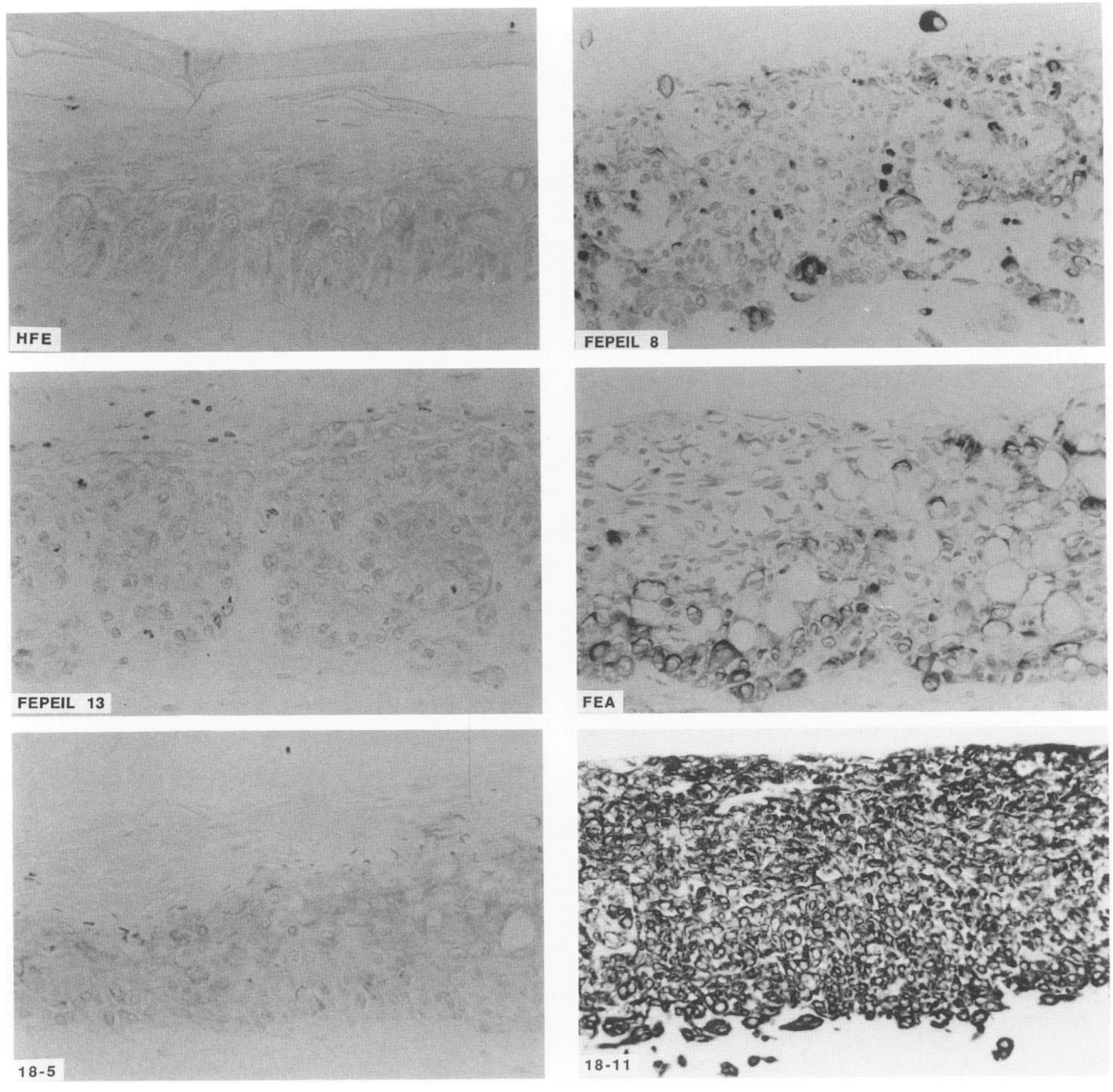

Figure 3. *Cross section of methanol-fixed, paraffin-embedded organotypic cultures of HFE and HPV immortalized keratinocytes. Immunohistochemistry of cytokeratin K8 expression with methyl green counterstain, original magnification, ×200.*

18-11 cell line and the others. Thus it appears that this pattern of PCNA expression is a feature shared by all HPV-immortalized cell lines regardless of their differentiation capacity.

Discussion

The expression of four different markers of differentiation and proliferation were studied in several HPV 16 or 18 immortalized cell lines to assess how these cellular proteins are affected by HPV, and to what extent these changes may be associated with premalignant or malignant epithelial lesions. The expression of filaggrin and keratins K1 and K8 were studied to assess each cell line's epithelial differentiation capabilities. The variety of patterns of expression of these proteins yielded some interesting correlations with morphologic appearance and tumorigenic potential, suggesting that immortalization of keratinocytes by HPV can be associated with a variety of differentiation capabilities. The proliferation-associated protein PCNA provided a uniformly unique pattern of expression that distinguished all HPV-immortalized cell lines from normal keratinocytes, however.

Normal epithelia *in vivo* and our organotypic cultures of normal keratinocytes express PCNA only in the cells of the basal layer[46] (A. Gown unpublished results). Organotypic cultures of all of the HPV-immortalized cell lines show strong PCNA expression throughout the entire thickness of the epithelial layer, indicating that regulation of PCNA expression is abnormal in these cells. Commit-

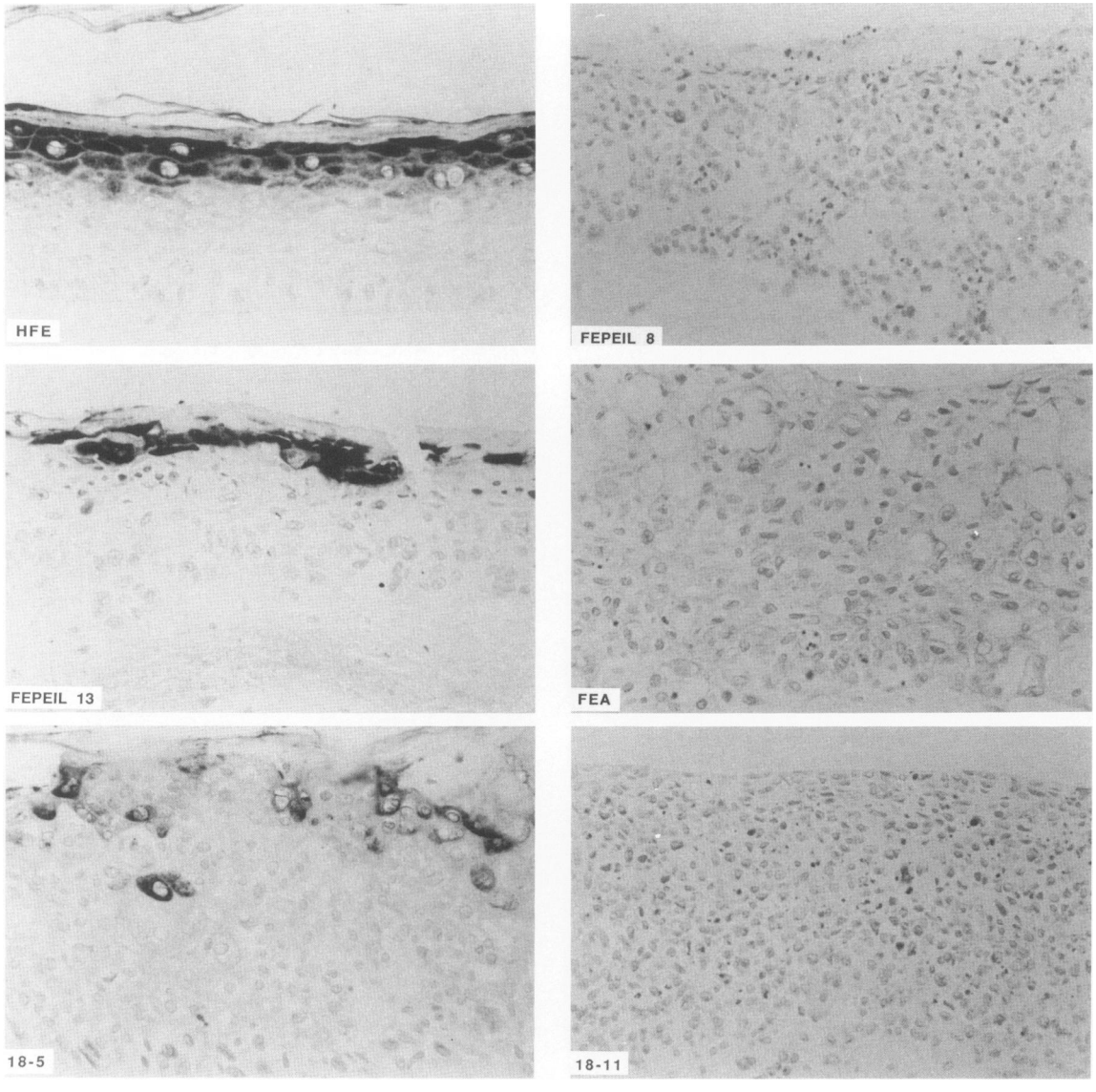

Figure 4. *Cross section of methanol-fixed, paraffin-embedded organotypic cultures of HFE and HPV immortalized keratinocytes. Immunohistochemistry of filaggrin expression with methyl green counterstain, original magnification, ×200.*

ment of normal epithelial cells to terminal differentiation is accompanied by a block of further cell division.[49,50] Our results suggest that maintenance of proliferative activity is a consequence of immortalization of epithelial cells by HPV types 16 or 18. Interestingly maintenance of proliferative activity in these cell lines does not preclude terminal differentiation, as can be seen with the cornifying FEPEIL 13 and 18-5 cell lines. Thus it appears that, regardless of the morphology of the associated lesion, HPV sequences confer the ability to maintain cell division after this property is normally lost in epithelia.

Such a role for HPV seems both plausible given known HPV gene interaction with cellular proteins,[7,8] and reconcilable with the multi-step theory of malignant progression. An hypothesis that sequences from HPV 16 and 18 affect cell cycle control is consistent with the fact that E6 and E7 proteins from these HPV types have been shown to form potentially inactivating complexes with cellular p53 and Rb proteins, respectively.[7,8] Although less is known about p53, it resembles Rb protein in many ways,[51] and Rb protein is strongly suspected of playing a role either in the entry of cells to G_0 or preventing them from making the transition from G_1 to the S phase of the cell cycle.[9,52–54] Maintenance of proliferative activity could contribute to the multistep carcinogenic process by allowing epithelial cells to go through an abnormally high number of mitoses, increasing the chances that a secondary tumorigenic event would occur.

The possibility that a secondary event also could be responsible for the aberrant pattern of PCNA expression in our HPV-immortalized cell lines cannot be ruled out because all of the cells used in this study were of late

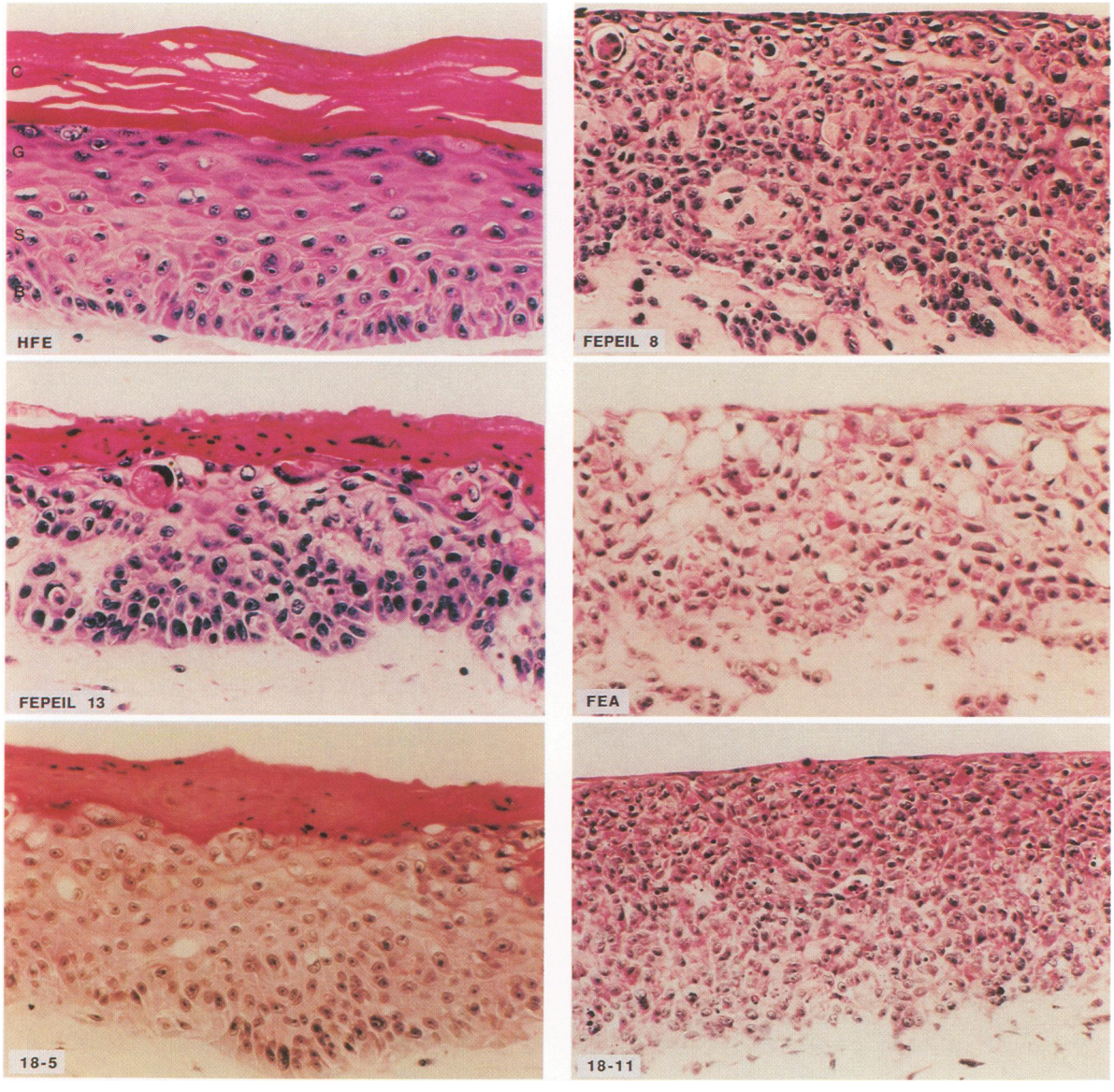

Figure 5. *Cross section of methanol-fixed, paraffin-embedded organotypic cultures of HFE and HPV immortalized keratinocytes. Immunohistochemistry of PCNA expression with methyl green counterstain, original magnification, ×200.*

passage (passage 60 or greater). A number of observations, however, lend support to the concept that this PCNA expression is a consequence of HPV immortalization rather than of some subsequent event. The immortalized cell lines show a variety of phenotypes for each of the characteristics that have been studied. Morphologically they present a spectrum of differentiation capabilities,[22] and the expression of all of the markers except PCNA fail to show a consistent pattern in each of the cell lines. Furthermore the pattern of PCNA expression seen in the organotypic cultures of all of the HPV-immortalized cell lines is distinct from that seen in HFEs grown on organotypic culture, and thus suggests that this aberrant PCNA expression could be a consequence of HPV-immortalization.

Human papilloma virus–immortalized cell lines display a variety of differentiation potentials in organotypic culture, and some interesting correlations between marker expression and the organotypic morphology and tumorigenic potential of these cell lines were observed. All three of the FEPEIL 8, FEA, and 18-11 cell lines lack cornification on organotypic cultures, and the undifferentiated morphology of their epithelial layers resembles that of a carcinoma-*in-situ* lesion. Filaggrin expression is uniformly absent in all of these cell lines, whereas it is present in the normal and more differentiated FEPEIL 13 and 18-5 organotypic cultures. This suggests that loss of filaggrin expression may be a common characteristic of undifferentiated epithelia. *In vivo* evidence to support this idea has been presented in studies of both oral and cer-

vical lesions.[27,28] In these studies, lack of filaggrin expression was reported in oral carcinoma-*in-situ* lesions, and significant decreases in filaggrin expression were associated with increasing grade of cervical intraepithelial neoplasia (CIN). Of particular interest in the study of cervical lesions was the fact that the majority of HPV 16 and 18 associated CIN III lesions expressed only trace amounts of filaggrin.[27] Our results provide *in vitro* corroboration of lost filaggrin expression in association with high-grade HPV 16 or 18 associated dysplasia, suggesting a potentially useful parameter in determining severity of epithelial lesions. These observations become more important when considering that an increased rate of progression to tumorigenicity has been directly associated with higher grades of CIN.[19]

Another interesting result observed in this study was the extremely high level of expression of keratin K8 by the tumorigenic 18-11 cell line when grown on organotypic cultures. This becomes particularly interesting when the results of a number of recent studies of a variety of squamous cell and other carcinomas are considered. Several studies of squamous cell carcinomas of the cervix have reported abundant levels of K8 expression,[25,55–57] and expression of K8 in breast carcinomas derived from ductal epithelia that do not normally express K8 also has been reported.[58] A recent report has even described aberrant K8 expression in the majority of several different sarcomas that were studied.[59] Extrapolation of our results to potentially useful markers in the evaluation of cervical, breast, or other disease is inhibited by the fact that our studies use cell lines established from epidermal cells; however, our results do provide an example of another tumorigenic cell line that shows strong K8 expression where it is normally unexpressed. In addition, weak K8 expression has been reported in CIN III lesions, whereas CIN I and CIN II lesions in the same study were found to be negative.[26] This latter result agrees with our observation of weak K8 expression in organotypic cultures of the FEA and FEPEIL 8 cell lines, whose undifferentiated morphologies resemble the morphology of such high-grade epithelial lesions. Theories regarding K8 expression in cervical malignancies have included the idea that the malignancies may be derived from cells that normally express K8[55]; or that the differentiation program of the malignant cells has changed toward one resembling that of a simple epithelial cell[60]; or that a reversal of the developmental process has occurred in these cells because embryonic epidermis is initially a simple epithelium that later stratified.[60,61] In our study, the parental cells used to produce our cell lines were derived from neonatal human foreskin epidermis, which does not normally express K8. Thus we have found that a change in the differentiation program of epithelial cells could be responsible for producing such an aberrant phenotype, and that K8 expression can occur in malignant cells derived from tissues that do not normally express K8.

Our results suggest that HPV sequences have an effect on normal control of proliferative activity, and that this could be an important factor in the progression of HPV-associated epithelial lesions. Interestingly this maintenance of proliferative potential was seen in cell lines that cornified in organotypic culture and produced epithelial layers that showed some degree of differentiation, thus raising the possibility that all HPV 16 and 18 related lesions regardless of morphologic severity have the potential to progress. Experiments addressing expression of epithelial differentiation markers did, however, reveal phenotypic alterations that appear to be associated with more advanced epithelial lesions; lesions that might be appropriately considered malignant or premalignant and more likely to progress to malignancy than less dysplastic lesions. More *in vivo* studies and organotypic experiments with HPV-immortalized cervical cells should help establish whether alterations in expression of markers like K8 and filaggrin could be useful in the evaluation of HPV-related disease, and should help define the effect of HPV sequences on the control of cellular proliferation.

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